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Abstract

Natural *Saccharomyces cerevisiae* yeast strains exhibit very large genotypic and phenotypic diversity. However, the link between phenotype variation and genetic determinism is still difficult to identify, especially in wild populations. Using genome hybridization on DNA microarrays, it is now possible to identify single-feature polymorphisms among divergent yeast strains. This tool offers the possibility of applying quantitative genetics to wild yeast strains. In this instance, we studied the genetic basis for variations in acetic acid production using progeny derived from two strains from grape must isolates. The trait was quantified during alcoholic fermentation of the two strains and 108 segregants derived from their crossing. A genetic map of 2212 markers was generated using oligonucleotide microarrays, and a major quantitative trait locus (QTL) was mapped with high significance. Further investigations showed that this QTL was due to a nonsynonymous single-nucleotide polymorphism that targeted the catalytic core of asparaginase type I (*ASP1*) and abolished its activity. This QTL was only effective when asparagine was used as a major nitrogen source. Our results link nitrogen assimilation and CO_2 production rate to acetic acid production, as well as, on a broader scale, illustrating the specific problem of quantitative genetics when working with nonlaboratory microorganisms.

Introduction

Yeasts used in wine fermentation are derived from wild strains capable of developing in grape juice. Genomic and genetic analyses have shown huge variability among this population (Bidenne *et al.*, 1992; Hennequin *et al.*, 2001; Winzeler *et al.*, 2003; Dunn *et al.*, 2005; Legras *et al.*, 2005). This genetic variability is correlated with metabolic variations (Soles *et al.*, 1982; Giudici & Zambonelli, 1992; Remize *et al.*, 2000a, b; Shinohara *et al.*, 2000; Murat *et al.*, 2001), which are largely heritable (Marullo *et al.*, 2004). Metabolic pathways have been extensively dissected in *Saccharomyces cerevisiae*, usually by analyzing laboratory-induced recessive monogenic mutations (Rose & Harrison, 1989). Although the enzymes identified are essential for deciphering cell biochemistry, they do not fully explain metabolic fluctuations among individuals. First, natural isolates, which are generally prototrophs, exhibit wide variations in their secondary metabolite production. Second, metabolic parameters generally vary in a complex, continuous way, and not as all-or-nothing switches (Hatzimanikatis *et al.*, 1998). This can be attributed to a typical polygenic determinism (Romano *et al.*, 1985; Giudici & Zambonelli, 1992; McCusker *et al.*, 1994; Marullo *et al.*, 2004). The underlying genetic determinisms among the wild population may be detected, at least in part, by quantitative trait locus (QTL) mapping (Lander & Botstein, 1989; Lynch & Walsh, 1998). Two main advantages of this approach are as follows: (1) no *a priori* hypothesis on gene function and sequence variation is required; and (2) it is often capable of detecting multiple genes that affect the value of a single quantitative trait. *Saccharomyces cerevisiae* provides an ideal framework for QTL analysis, due to its high recombination rate, its richly annotated genome, and the fact that genes can be directly manipulated in their genomic context. In addition, DNA microarrays provide a rapid method for generating dense

genetic maps, thus facilitating efficient genetic mapping (Winzeler *et al.*, 1998; Steinmetz *et al.*, 2002; Deutschbauer & Davis, 2005). In this study, we analyzed the production of acetic acid as a specific metabolic quantitative trait. Acetic acid is one of the most volatile organic compounds in wine, and constitutes a major organoleptic defect at concentrations above 0.6 g L^{-1} (Riberau-Gayon *et al.*, 2000). Acetic acid is mainly produced by yeast during alcoholic fermentation, and the choice of strain has a major impact on this production (Giudici & Zambonelli, 1992; Romano *et al.*, 1994; Remize *et al.*, 2000a, b; Marullo *et al.*, 2004). The metabolic pathway of acetate under fermentation conditions mainly involves acetaldehyde dehydrogenase (ALD) (Remize *et al.*, 2000a, b). Five ALD isoforms have now been identified in *S. cerevisiae*. Three of them are cytosolic (encoded by *ALD6*, *ALD2*, and *ALD3*), and the other two are mitochondrial (encoded by *ALD4* and *ALD5*) (Navarro-Avino *et al.*, 1999). The major isoforms involved in winemaking are Ald6p and Ald5p. However, Ald4p and an unknown alternative pathway may compensate for the absence of these enzymes (Saint-Prix *et al.*, 2004). Although this pathway has been described in detail, most yeast strains have functional ALD genes and present wide phenotypic differences, indicating that other genetic causes have not yet been elucidated. ALD isoform compartmentalization and cofactor specificity (NAD+ or NADP+) (Saint-Prix *et al.*, 2004), the redox relationship between acetate and glycerol production under high-osmolarity conditions (Blomberg, 2000) and other unidentified parameters make acetic acid production a typical quantitative trait.

In this study, we proposed to apply a QTL mapping strategy to investigate the genetic determinism of acetic acid production in wine fermentation. Using progeny derived from two parental strains with noticable differences in acetic acid production, and originating from wine starters, we aimed to investigate the genes and related allelic variations determining their phenotype differences. This work was divided into four main phases. First, phenotypic data were collected from a large progeny derived from the two parental strains. Second, numerous molecular genetic markers between parental strains were defined and used to genotype some of the phenotyped progenies. This step was carried out by comparative genome hybridization using DNA microarrays. Third, QTLs were mapped, establishing a statistical link between trait value and molecular genetic markers. Four, relying on the powerful genetics of *S. cerevisiae*, a mapped QTL was molecularly dissected and validated by molecular engineering.

Materials and methods

Media and culture conditions

Saccharomyces cerevisiae was grown in YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} Bacto peptone, 20 g L^{-1} glucose) at 30 °C. Sporulation was induced on ACK medium (10 g L^{-1} potassium acetate) at 24 °C. Transformed yeast strains were selected and grown on YPD medium supplemented with 100 μg mL^{-1} G418 (Sigma, Lyon, France) or 200 μg mL^{-1} nourseothricin (Werner Bioagents, Iena, Germany) (Goldstein & McCusker, 1999). Cells for asparaginase I assays were cultivated in SD (yeast nitrogen base with 6.7 g L^{-1} ammonium sulfate and 20 g L^{-1} dextrose). All media were supplemented with 20 g L^{-1} agar for solid culture. Gene transplacement (pop-out) was selected by inducing colony growth on MA-ASN medium [1.7 g L^{-1} yeast nitrogen base without amino acids and ammonium sulfate, 20 g L^{-1} glucose, 4.8 g L^{-1} methylamine hydrochloride (Sigma, Lyon, France), and 0.6 g L^{-1} L-asparagine, pH 6.5]. Methylamine acts as a gratuitous inducer of nitrogen

Table 1. Yeast strains used

Strain	Genetic background	Genotype	Origin
BY4742	S288c	BY4741 *Mat a his3Δ1 leu2Δ0 met15Δ5 ura3Δ0*	Euroscarf
BY-ΔASP1	S288c	BY4742 *asp1::KanMx4 Mat a his3Δ1 leu2Δ0 met15Δ5 ura3Δ0*	Euroscarf
SB	SB	*HO/HO, asp1-H142/asp1-H142*	Marullo *et al.* (2006)
S9	SB	*HO/ho::KanMx4, asp1-H142/asp1-H142*	Marullo *et al.* (2006)
ho-SB	SB	*ho::kanMx4, asp1-H142*	Marullo *et al.* (2006)
ho-SB-T8	SB	*ho::kanMx4, asp1::pPM4*	This study
ho-SB-wt	SB	*ho::kanMx4, ASP1-wt*	This study
ho-SB-Δ	SB	*ho::kanMx4, Δasp1::pPM2-NAT*	This study
SB-Δ/wt	SB	*ho::KanMx4/ho::KanMx4, Δasp1::pPM2-NAT/asp1-H142*	This study
SB-H142/wt	SB	*ho::KanMx4/ho::KanMx4 ASP1-wt/asp1-H142*	This study
SB-wt/wt	SB	*ho::KanMx4/ho::KanMx4 ASP1-wt/ASP1-wt*	This study
GN	GN	*HO/HO, ASP1-wt/ASP1-wt*	Marullo *et al.* (2006)
GN-Δ/wt	GN	*HO/HO, Δasp1::pPM2-NAT/ASP1-wt*	This study
GN-Δ/Δ	GN	*HO/HO, Δasp1::pPM2-NAT/Δasp1::pPM2-NAT*	This study
BN	BN	*hybrid ho-SB* and *GN ho::KanMx4/HO, asp1-H142/ASP1-wt*	Marullo *et al.* (2006)

catabolic repression (NCR) (Salmon & Barre, 1998) and represses the synthesis of asparaginase type II activities. In our context, this medium selects strains carrying a functional cytosolic asparaginase (type I).

Yeast strains used for QTL mapping

The base material for our study of enological properties consisted of two commercial winemaking strains, Bo213 and VL1 (Laffort Oenologie, Bordeaux, France) isolated from natural yeast populations. Two meiotic spore clones derived from Bo213 and VL1, known as SB and GN, respectively, were defined as parent strains. Both were diploid homothallic. The homothalism of SB was bypassed to make it possible to work with haploid strains. One *HO* allele of the SB diploid strain was deleted to obtain the S9 diploid (*HO/ho::KanMx4*) and its derivative meiotic haploid clone (*MATα, ho::KanMx4*) ho-SB. A GN haploid spore (*HO*) was crossed with ho-SB (*ho::KanMx4*) by micromanipulation to produce the BN hybrid. The construction of these strains has been described elsewhere (Marullo *et al.*, 2006). A large meiotic progeny clone population derived from the BN hybrid was obtained by sporulation. All the yeast strains used are listed in Table 1.

Plasmid construction

All plasmids are listed in Table 2. The pPM1 plasmid was obtained by ligation of the largest fragment of pFVL99, cut by BamHI and BglII. The disruptive plasmid pPM2 was constructed by cloning the central portion of the *ASP1* gene into the SacII and XhoI restriction sites of pFVL99. Restriction sites were incorporated into the 5′ and 3′ ends of the *ASP1* fragment by PCR, using genomic DNA from the GN strain as a template and oligonucleotides p227 5′-TCCCCGCGGGGAAAATCTTGGGTACCGGTGG-3′ and p228 5′-CCGCTCGAGCGGTCAGTTTGAATTTGTGGCA-3′. The pPM3 plasmid was obtained by cloning the *ASP1* gene produced by PCR using GN genomic DNA as a template with oligonucleotides p225 5′-TGACGATGACTTTGTTGAA-3′ and p226 5′-TACAAAATTTTCACCCACCAT-3′ (80 nucleotides before ATG to five nucleotides after the stop codon) in the TOPO 2.1 cloning vector kit (Invitrogen, Carlsbad, CA). The integrative plasmid pPM4 was constructed by cloning the small fragment of pPM3 into the SpeI and NotI restriction sites of pPM1.

Engineered parent strain construction for *ASP1* validation

First, the wild-type allele of *ASP1* was introduced into the ho-SB strain using the pPM4 plasmid linearized by cutting the *ASP1* ORF by BglII. Nine nourseothricin-resistant clones containing two tandem copies of *ASP1* were obtained. As the BglII recombination site is located upstream of *asp1-H142*, the original mutated H142 allele had no promoter and *ASP1-wt* had the endogenous promoter. Surprisingly, these clones did not have any asparaginase activity. This may be due to inefficient mRNA synthesis or 3′ processing of the *ASP1-wt* copy because of the proximity of the NAT marker. To restore the native structure of the whole gene, we selected a gene transplacement event (Scherer & Davis, 1979) to eliminate the *NAT* marker, and the original *asp1-H142* copy, by selecting clones growing on asparagine as sole nitrogen source in the presence of methylamine (MA-ASN). This was possible because asparaginase activity is needed for growth on this medium. The gene transplacement resulted in expression of the *ASP1-wt* allele, making it possible to select the desired clones on this specific medium. Once the *ASP1-wt* allele had been introduced into ho-SB, strains SB-H142/wt and SB-wt/wt, bearing one or two allele copies of GN, respectively, were derived by successive crosses. Second, *ASP1* was disrupted in the GN background, using the pPM2 plasmid cut in the *ASP1* ORF by XbaI. One nourseothricin-resistant clone (GN-Δ/wt) proved to contain the expected truncated copy of *ASP1*. After sporulation, homozygous GN (*asp1-Δ/asp1-Δ*) was recovered and named GN-Δ/Δ. The *bona fide* structure of the locus in all constructed strains was verified by PCR and sequencing.

Genotyping using high-density oligonucleotide microarrays

Genomic DNA was isolated, fragmented, labeled, and hybridized to Affymetrix S98 microarrays, as previously described (Winzeler *et al.*, 1998). We applied this procedure to three independent ho-SB cell cultures, three independent GN cultures, and one culture of each of the 48 segregants to be genotyped. We then selected biallelic markers using the

Table 2. Plasmids used in this work

Plasmid	Description	Origin
pFvL99	Integrative, NAT^R	F. Van Leuveeren
pPM1	Integrative, NAT^R, pFvL99 without BglII restriction site (excision of BamHI/BglII small fragment)	This study
pPM2	Integrative, NAT^R, central portion of *ASP1* cloned in pFvL99 (SacII/XhoI)	This study
pPM4	Integrative, NAT^R, *ASP1* ORF (80 before ATG to five after stop) cloned in pPM1 (SpeI/NotI)	This study

same statistical procedure as Brem *et al.* (2002). First, probes identified as low perfect-match (PM) vs. mismatch (MM) hybridizers to S288c (BY) genomic DNA were discarded (Brem *et al.*, 2002). All further analyses, except the chromosome IX aneuploidy test, were performed on log (PM/MM) values. For each microarray, data were divided by their median for normalization. Using the Z and z statistics described in Brem *et al.* (2002), we selected 35 392 probe pairs that had high hybridization differences between ho-SB and GN. A second test, based on a clustering algorithm, was used to select 7167 of these probe pairs for evidence of 2 : 2 segregation in the segregating population. Genotypes were inferred from cluster assignments as described in Brem *et al.* (2002). We then removed markers with < 8 instances of the same genotype (highly unlikely by random segregation) and probe pairs for which genotype inference failed in over 15 hybridizations. Finally, probe pairs corresponding to Ty elements were removed, as their locations on the ho-SB and GN genomes were unknown. To examine the ploidy of chromosome IX, we considered raw log (PM) values of the three ho-SB and three GN experiments. For each probe, the average difference Δ between ho-SB and GN values was computed, and the distribution of these average differences in chromosome IX probes was compared to the distribution in all probes of the genome.

Search for relationships between markers and acetic acid production

We tested for associations between genotypes and acetic acid production as follows. At every marker position, segregants were separated into two groups according to their genotype at the marker. The difference in phenotype values between the two groups was then tested by the Wilcoxon–Mann–Whitney (WMW) test. This test was preferred to Student's *t*-test or regression, to avoid normality issues. Genome-wide significance was addressed by permuting segregant phenotypes and rescanning the genome.

Sequencing and restriction fragment length polymorphism (RFLP) fine mapping

Both strands were sequenced by Millegen (Toulouse, France). Primer sets generating 500–800-bp fragments of the following genes were used: *YDR316w* p136, 5′-GTTATTAAAGCCAGATGGAAG-3′, and p137, 5′-TTTACACGATTCTAAGCCGT-3′; *YDR317w* p114, 5′-TGCGGAGAACTCAGGTACCAA-3′, and p115, 5′-CTTTTCCTTTCAATCGCCATC-3′; *YDR320c-A* (*DAD4*) p116, 5′-TTTCCTTGGATGCAGATGCA-3′, and p117, 5′-AACAAAGCAGATAGGGAGGA-3′; *YDR321w* (*ASP1*) p124, 5′-TCATGTTGACCTGACCATCCA-3′, and p125, 5′-GGCTTAGATAACCAGATGCGA-3′; *YDR322w* (*MRPL35*) p145, 5′-CAATACCAGGCAATATCTAGT-3′, and p146, 5′-CAGAAAGGGTGTGATCCAAA-3′. RFLP analysis of PCR products was carried out using the following restriction enzymes – *YDR316w*, Ksp632 cutting SB allele; *YDR317w*, NlaIII cutting GN allele; *YDR321w*, Hin4I cutting GN allele; and *YDR322w*, AccI cutting GN allele.

Acetic acid production assay in alcoholic fermentation

Fermentation experiments in order to measure acetic acid production were carried out in ASN medium containing 200 g L^{-1} glucose/fructose, previously referred to as Model Synthetic Medium (Marullo *et al.*, 2004). The nitrogen source was 190 mg (N) L^{-1}: two-thirds asparagine and one-third ammonium. Progeny, parent strain and hybrid values were the means of three, 30 and nine experiments, respectively. Acetic acid (expressed in g L^{-1}) was determined chemically by colorimetry ($OD_{460\,nm}$) in continuous flux (Sanimat, Montauban, France).

CO_2 production rate measurement

The amount of CO_2 released (g L^{-1}) and the CO_2 production rate (g L^{-1} h^{-1}) were determined by automatic measurement of bioreactor weight loss at 20-min intervals (Marullo *et al.*, 2006).

Asparaginase assay

Cell-free extracts were prepared as follows. Two hundred and fifty microliters of yeast cell culture was harvested by centrifugation and washed twice with 25 mM potassium phosphate (pH 7.5). Glass beads (diameter 0.5 mm) were added, and cells were disrupted by vortexing three times for 1 min at 4 °C. Cell debris was removed by centrifugation (5 min at 7500 ***g***). The supernatant was immediately used to assay asparaginase activity, as described by Dunlop & Roon (1975).

Results

Analysis of acetic acid production in parent strains and segregating population

Acetic acid production for parent strains is reported in Table 3. The initial diploid SB and GN parents were high producers (0.63 g L^{-1}) and low producers (0.20 g L^{-1}), respectively. In the S9 strain, the disruption of the *HO* allele by the *ho*::*KanMx4* cassette did not change acetic acid production as compared to the original SB strain. However, haploid or diploid status influenced acetic acid production, as illustrated by the comparison of S9 diploid and ho-SB haploid strains. This ploidy effect led us to analyze only haploid progenies for further QTL mapping.

Table 3. Acetic acid production of strains ho-SB, S9, SB, GN, and hydrid BN

Strain (n = repetitions)	Genotype	Acetic acid production (g L^{-1})
ho-SB ($n = 30$)	*ho::KanMx4*	0.80 ± 0.08
S9 ($n = 6$)	*ho::KanMx4/HO*	0.61 ± 0.04
SB ($n = 18$)	HO/HO	0.63 ± 0.06
GN ($n = 30$)	HO/HO	0.20 ± 0.03
BN ($n = 6$)	*ho::KanMx4/HO*	0.65 ± 0.06

Acetic acid production is expressed as means $\pm$ SD of n determinations after complete fermentation in ASN medium.

The high value of the BN hybrid (identical to that of SB) showed that there is a nonadditive mode of inheritance for most of the QTLs responsible for high production. The 108 haploid progenies in the segregating population were analyzed (Fig. 1). Acetic acid production values were roughly normally distributed, supporting polygenic determinism of the phenotype. The progeny values ranged from 0.11 to 1.46 g L^{-1}, with 22% of the population showing values outside the parental range. This transgressive segregation indicated that alleles with opposite effects from both parents could affect the phenotype.

Marker map construction

The selective genotyping of individuals taken in tail distribution increases the QTL detection power, as demonstrated both theoretically (Lander & Botstein, 1989) and experimentally (Ayoub & Mather, 2002). We selected 48 segregants on the basis of the highest and lowest phenotypic values for acetic acid production. To avoid biased samples in QTL mapping, segregants were selected from independent tetrads. Their genomic DNA was hybridized on high-density oligonucleotide microarrays for genotyping (Winzeler *et al.*, 1998). First, oligonucleotides showing differential signals between GN and ho-SB were primarily selected by hybridizing genomic DNA from both strains on microarrays in triplicate. Second, the entire hybridization dataset of the parents and 48 segregants were used to generate a marker map, by applying the algorithms described in 'Materials and methods'. A dense coverage of the genome was obtained, with 2212 reliable markers (Fig. 2). Microarray-derived markers were represented using the physical map of the S288c reference strain.

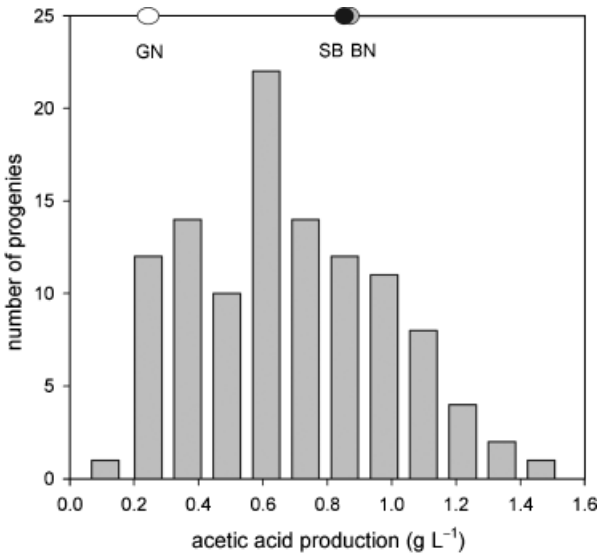

Fig. 1. Distribution of acetic acid production levels in BN progeny ($n = 108$). Parent GN, SB and hybrid BN mean values are shown on the edge as white, black and gray dots, respectively.

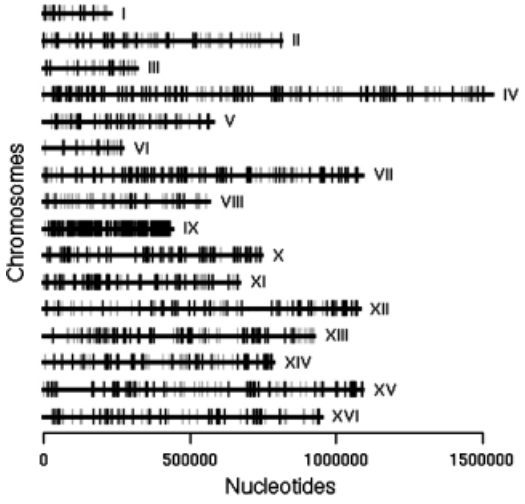

Fig. 2. Microarray-derived marker map. The genotype of one segregant is presented. Each vertical tick represents one genetic marker; GN and SB inheritance are shown in gray and black, respectively.

Identification of chromosome IX aneuploidy in the ho-SB strain

Surprisingly, an unexpectedly high number of markers (17%) was found on chromosome IX, representing only 4% of the S288c genome. Fragments covering some of these markers were amplified and sequenced to determine whether this density reflected true enrichment of sequence polymorphisms between GN and ho-SB. We found poor validation (one polymorphism out of six sequences) in comparison to markers from other chromosomes (13 out of 16). This strongly suggests that numerous chromosome IX markers were obtained from aberrant hybridization signals. These aberrations could be due to aneuploidy if one parent strain had more than one copy of chromosome IX. In this case, half the segregants would inherit this additional chromosome, and hybridization signals would

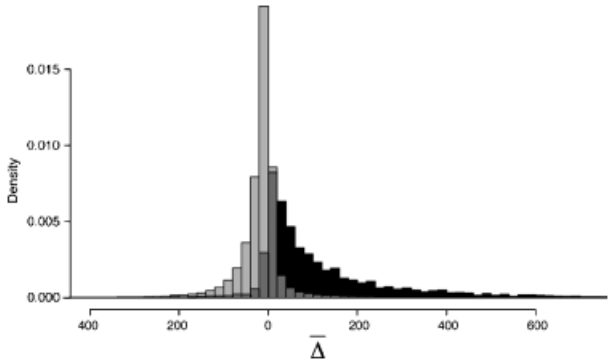

Fig. 3. Chromosome IX aneuploidy. For each probe on the array, Δ was calculated as the difference between the log(PM) signals obtained from one hybridization of ho-SB genomic DNA and one hybridization of GN genomic DNA. Three independent Δ values were averaged. The light gray histogram shows the distribution of mean Δ values in all 110 979 probes of the array, and the black histogram corresponds to their distribution in the subset of 3430 probes representing chromosome IX sequences. Dark gray: superposition. The two distributions differed with high significance ($P < 10^{-15}$, Kolmogorov–Smirnov test).

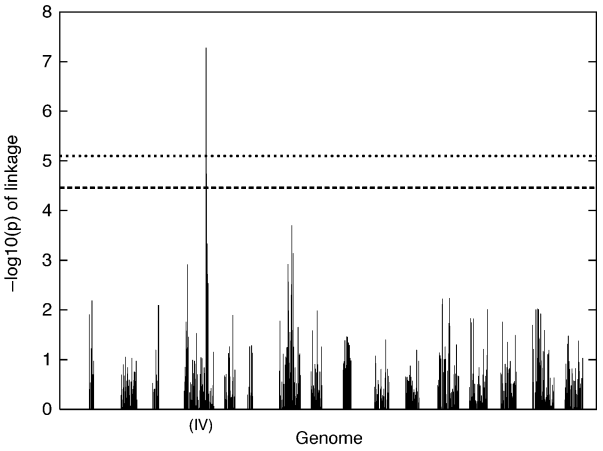

Fig. 4. QTL mapping of acetic acid production. The physical genome is represented on the *x*-axis, with gaps between consecutive chromosomes. Each peak corresponds to the nominal *P*-value of linkage between a marker located in the *x* position and acetic acid production. The two significance levels mentioned in the text are indicated by dashed lines. A QTL is mapped on chromosome IV.

be affected by gene copy numbers. Supporting this hypothesis, pulsed-field gel electrophoresis showed that the size of chromosome IX was different in GN and ho-SB, and that a few segregants had both types of chromosome IX (Marullo *et al.*, 2006). We also re-examined the microarray data, and found that hybridization intensities of probes interrogating chromosome IX sequences were consistently higher in ho-SB than in GN (Fig. 3). We concluded that ho-SB had two or more copies of chromosome IX, and that the high density of markers obtained for this chromosome was an artefact due to aneuploidy.

Identification of a locus involved in acetic acid overproduction

We then used these genotypes to map QTLs for acetic acid production. The relationship between trait values and genotype was tested at every marker position by splitting the segregants according to their allelic genotype (ho-SB or GN) and applying the WMW test for phenotypic difference between the two groups. We found only one linkage peak (nominal *P*-value = 5.25×10^{-8}) for a marker located on chromosome IV, in position 1 108 286 (bp) on the reference S288c genome (Fig. 4). We addressed significance empirically by permuting segregant indexes in a random manner and rescanning for QTLs. Among 1000 permutation tests, only 10 genome scans detected a locus at $P < 3.4 \times 10^{-5}$, and no locus was ever detected at $P < 8 \times 10^{-6}$. This indicated that our QTL detection was highly significant genome-wide. In order to refine the QTL mapping, we designed additional markers for the region by PCR and sequencing. We found four markers (located in *YDR316w*, *YDR137w*, *YDR320C-A*, and *YDR322w*) harboring RFLP on PCR products, and used them to genotype a total of 93 segregants. The linkage score improved to a *P*-value = 3.4×10^{-9}, and the region with highest linkage was narrowed down to 15 kb.

Identification of *ASP1* as the most probable candidate in the QTL

In order to identify the gene(s) underlying the QTL, we initially examined the *S. cerevisiae* database (www.yeastgenome.org/). In the 15-kb mapped region, 10 genes were annotated. Besides hypothetical ORFs (*YDR319c*) that we did not consider any further, four genes were attributed to nuclear functions (*YDR317w*, *YDR318w*, *YDR324c*, and *YDR325w*), two to the vesicular network (*YDR320* and *YDR323c*), and two to mitochondrial complexes (*YDR322c* and *YDR322c-3*). None of these genes had any obvious relationship to acetate production, so we did not consider them as good candidates. The last gene, *YDR321w* (*ASP1*), encodes constitutive cytosolic asparaginase (Sinclair *et al.*, 1994). This enzyme is responsible for catabolizing asparagine into aspartate and ammonia. As the nitrogen source in the media used for acetic acid production measurement consisted of two-thirds asparagine, this gene was considered to be worth further investigation. We sequenced the GN and SB alleles of *ASP1*, including 200 bp upstream and downstream from the ORF. Only two single-nucleotide polymorphisms (SNPs) were detected in these 1.5-kb sequences. The first polymorphism was a synonymous T > C change at 471 (ORF position) in SB. The second polymorphism was a G > C change at position 424, resulting in the replacement

of aspartate 142 by histidine. In GN, the Asp1p protein sequence was identical to the S288c reference sequence, whereas in SB, it contained the new D142H sequence. To evaluate the potential functional impact of this change, we first aligned several asparaginase sequences (Fig. 5a). Alignment of bacterial asparaginases with those of the SB and GN sequences showed that the D142H substitution was localized in the highly conserved 'HGTDTM' catalytic motif of asparaginase (PROSITE entry: PD0COO132). The probable structural effect of this substitution was visualized by aligning four asparaginase structures using DEEP VIEW software (www.expasy.org/). The substitution of histidine for aspartic acid was predicted to affect the catalytic core of asparaginase, probably leading to abolition of the interaction between $-COO^-$ from the aspartate residue and $-NH_3^+$ from the substrate (Fig. 5b). If the SB allele was null or weak, this could account for an otherwise unexplained difference in growth between the parent strains. In fact, SB grew slower than GN in the medium used for phenotype measurement. All of these theoretical and experimental elements prompted us to consider that the *ASP1* gene was at least partly responsible for the QTL. In this hypothesis, all descendants (and not only the ones used for mapping) that inherited the D142H substitution would have a statistically high level of acetic acid production. We typed the D142H substitution in the entire population of 108 clones, and confirmed the expected linkage (Fig. 6). The widely scattered values suggested that other loci also affected the phenotype.

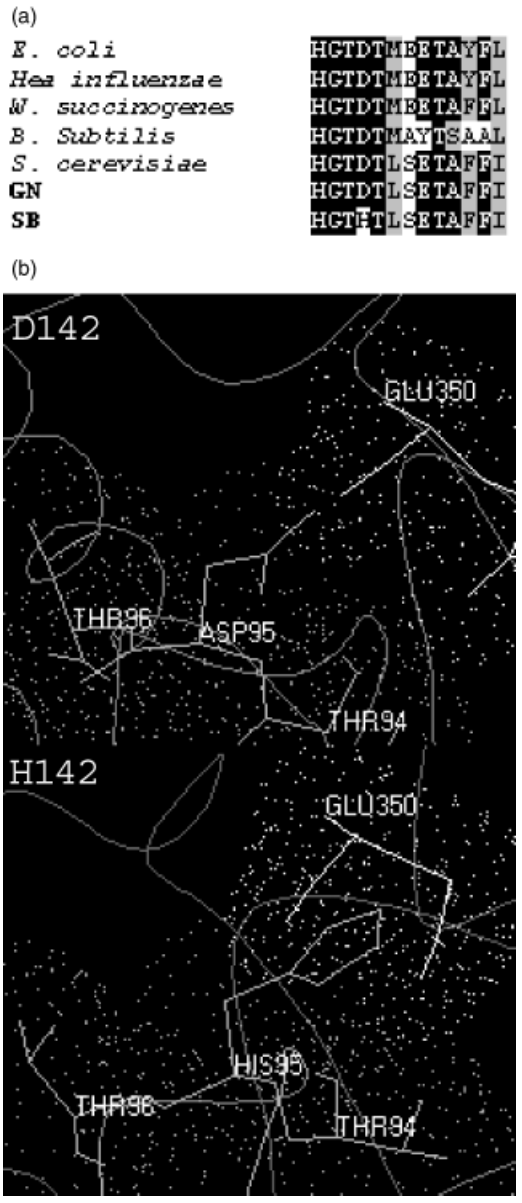

Fig. 5. Protein alignment of the catalytic domain of bacterial and yeast asparaginase I. (a) The D142H substitution of the SB parent strain is localized in the highly conserved motif HGTDTM. (b) Alignment of asparaginase structure with both GN (D142) and SB (H142) sequences. Residue surfaces of enzyme and substrate (GLU350) are represented by a dotted area.

Asparaginase I assay for parent strains and relative mutants

To determine the impact of the D142H mutation of *ASP1*, we engineered various alleles in the respective parent SB and GN (see Materials and methods). We obtained, for both backgrounds, strains presenting various *ASP1* alleles [Δ*asp1* (null), *ASP1-D142* (wild type), or *asp1-H142*] with homozygous or heterozygous status (Table 1). The asparaginase-specific activity of these strains was then enzymatically assayed. *Saccharomyces cerevisiae* incorporates the nitrogen of asparagine, due to two types of asparaginases. Type I, encoded by *ASP1*, is constitutive, cytosolic, and responsible for the main flux (Sinclair *et al.*, 1994). Other asparaginases, encoded by the *ASP3* cluster, are periplasmic and under NCR in ammonium-containing media (Dunlop & Roon, 1975; Kim *et al.*, 1988). Under our enzymatic assay conditions, only type I activity was measured and compared to that of laboratory control strains (BY and BY-ΔASP1). Table 4 shows that GN and control strain BY, both bearing the *ASP1-wt* allele (D142), presented high activity (4×10^{-2} IU), whereas SB (*asp1-H142/asp1-H142*) and the null mutant BY-ΔASP1 had no activity. As expected, asparaginase I activity was completely restored for engineered strains SB-wt/wt and absent in GN-Δ/Δ. Finally, heterozygous strains such BN (*ASP1-wt/asp1-H142*), GN-Δ/wt and SB-H142/wt had intermediate asparaginase I activity (65–35%), depending on the background of the strains.

Validation of *ASP1*'s role in the mapped QTL

The relationship between asparaginase I activity and acetic acid production was then analyzed in engineered strains.

Table 4. Asparaginase I activity for *ASP1* alleles

Strain (*ASP1* alleles)	Asp1p-specific activity (IU $\times 10^{-2}$)	Relative activity (GN = 100)
BY (*ASP1-wt*)	4.16 ± 0.31	96
BY-ΔASP1 (*asp1*::*KanMx4*)	ND	0
GN (*ASP1-wt/ASP1-wt*)	4.67 ± 0.4	100
GN-Δwt (*asp1*::*KanMx4/ASP1-wt*)	3.02 ± 0.8	65
GN-Δ/Δ*asp1*::*KanMx4/asp1*::*KanMx4*)	ND	0
SB-wt/wt (*ASP1-wt/ASP1-wt*)	4.15 ± 0.4	96
SB-H142/wt (*asp1-H142/ASP1-wt*)	1.15 ± 0.2	35
SB (*asp1-H142/asp1-H142*)	ND	0
BN (*asp1-H142/ASP1-wt*)	2.13 ± 0.15	45

Asparaginase activity is expressed in means ± SD of three different determinations.
ND, not detected.

Table 5. Control of acetic acid production by *ASP1* alleles

Strain	Asparaginase I relative activity	Acetic acid (g L^{-1})
GN (wt/wt)	100	0.19 ± 0.01
GN (Δwt)	65	0.21 ± 0.02
GN (Δ/Δ)	0	0.41 ± 0.03**
SB (wt/wt)	96	0.40 ± 0.04**
SB(H142/wt)	35	0.65 ± 0.05
SB(H142/H142)	0	0.63 ± 0.06
BN (H142/wt)	45	0.65 ± 0.06

Acetic acid production is expressed in means ± SD of five different determinations measured after complete fermentation in ASN medium. Asparaginase I relative activities are shown in Table 4.
**Statistically different from relative parent, ANOVA $\alpha = 0.01$.

These combinations were important for the assessment of the influence of other, unidentified loci (Fig. 6). The results are summarized in Table 5. First, in the GN background, the removal of asparaginase activity (*asp1*-Δ/*asp1*-Δ) led to the doubling of acetic acid production on ASN medium. On the same medium, the heterozygous strain (*ASP1-wt/asp1*-Δ), which had an intermediate level of asparaginase activity (65% of the wild type), produced the same low quantity of acetic acid as the original GN strain (*ASP1-wt/ASP1-wt*). This showed that, under these conditions, asparaginase I activity was actually responsible for low acetic acid production in the GN background. Moreover, in this case, the loss of Asp1p function was recessive in terms of acetic acid overproduction. Second, in the SB background, the homozygote (*ASP1-wt/ASP1-wt*) produced 30% less acetic acid than *asp1-H142/asp1-H142*. Asparaginase I activity was, therefore, only responsible for part of the acetic acid overproduction in this strain. SB alleles at other QTLs maintained a high level of acetic acid production, even in the presence of wild-type asparaginase I activity levels. Surprisingly, in the SB-H142/wt heterozygous strain, acetic acid production was the same as in the original SB strain. Unlike in the GN background, the loss of Asp1p function was dominant for acetic acid overproduction. This phenomenon may be due to the slightly lower asparaginase activity in the SB heterozygote (35% compared to 65% for the GN background). This phenomenon was not due to the different nature of nonfunctional alleles used (i.e. *asp1-H142* and *asp1*-Δ for SB and GN heterozygotes, respectively). In fact, in the SB background, SB-H142/wt and SB-Δ/wt showed the same acetic acid production level (data not shown). Moreover, the nonadditive effect of the *asp1-H142* allele on acetic acid overproduction was also verified in the BN hybrid, which showed the same acetic acid production in ASN medium as SB. All these findings suggested that at least one epistatic, recessive locus in the GN genome interfered with asparaginase I activity and, consequently, acetic acid overproduction.

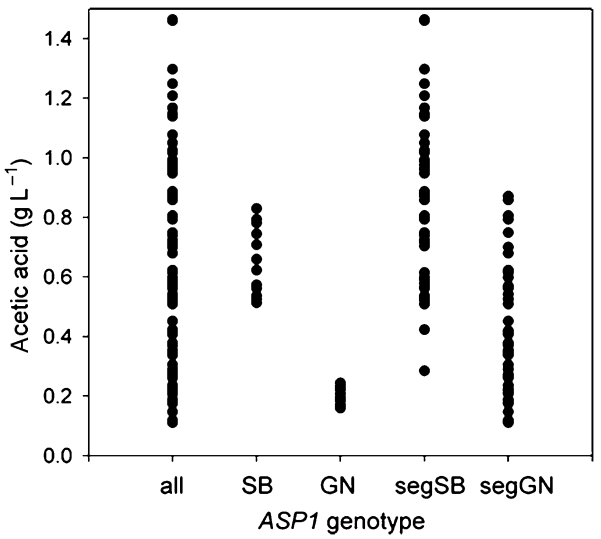

Fig. 6. Association between acetic acid production and genotype at the *ASP1* locus (nucleotide 424). The first column shows acetic production levels of 108 segregants. The second and third columns show acetic acid production of both parents (10 replicates). The fourth and fifth columns show acetic acid production of segregants that inherited the *ASP1* locus from SB and GN, respectively.

Nitrogen limitation of *asp1* mutants affected CO_2 production rate and acetic acid production in asparagine-containing medium

The relationship between limiting asparaginase activity and acetic acid production was then analyzed during fermentation for both parents (Fig. 7). As expected from QTL analysis, at the end of fermentation, strains lacking asparaginase I activity overproduced acetic acid in both backgrounds. About half of this overproduction was concomitant with a reduction in CO_2 flux in the first few hours of fermentation. The reduction in CO_2 flux was more

marked for the SB (Fig. 7a vs. b) than the GN background (Fig. 7c vs. d). This correlated with the overproduction of acetic acid by the SB and GN-Δ/Δ strains (+0.26 and +0.19 g L^{-1}, respectively). We defined three nitrogen depletion stages by monitoring asparagine and ammonium: stage 1 (dark gray), the medium contained ammonium and asparagine; stage 2 (light gray), ammonium was depleted and only asparagine remained; and stage 3 (white), the medium contained no more nitrogen sources. In strains carrying the *ASP1-wt* allele, stages 1 and 2 were simultaneous, whereas in *asp1* mutants (SB and GN-Δ/Δ), asparagine uptake (stage 2) continued after the complete depletion of ammonium. During this latter phase, both *asp1* strains showed both an increase in acetic acid production and a reduction in CO_2 flux. These findings suggested that overproduction of acetic acid was an indirect consequence of inefficient asparagine assimilation. To test the impact of asparagine on this phenomenon, asparagine was replaced as the nitrogen source for fermentation by an amino acid mixture without asparagine (KP medium) (Marullo *et al.*, 2006). First, marked acetic acid production differences were observed between the original SB (0.51 g L^{-1}) and GN (0.20 g L^{-1}) strains. This showed that the use of asparagine as the main nitrogen source was not the key factor in acetic acid production discrepancy between parent strains. Second, in this medium, the nature of the *ASP1* allele (wild type or null) was not correlated with acetic acid production (0.48 and 0.20 g L^{-1} for SB-wt/wt and GN-Δ/Δ respectively). This suggested that the QTL that we found was not effective when nitrogen sources did not contain asparagine.

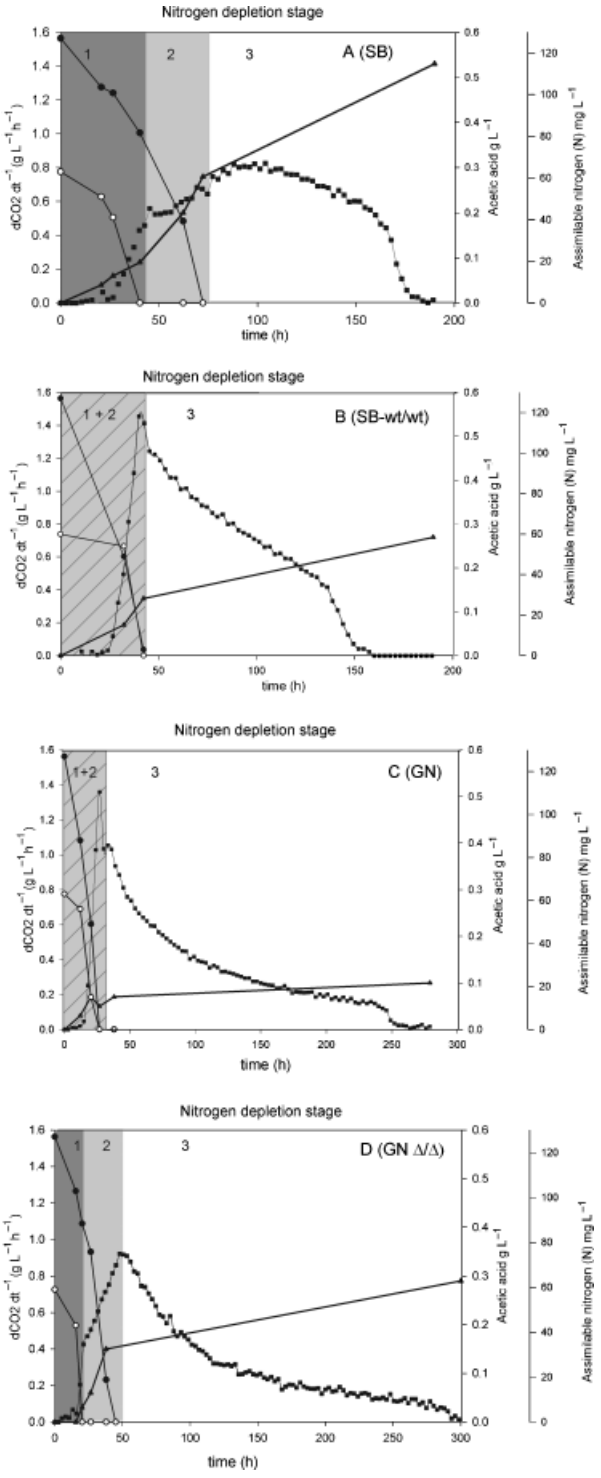

Fig. 7. Relationship between asparagine assimilation, acetic acid production, and CO_2 flux in parent backgrounds. CO_2 production rate (g L^{-1} h^{-1}) was measured for SB (*asp1-H142/asp1-H142*), SB-wt (*ASP1-wt/ASP1-wt*), GN (*ASP1-wt/ASP1-wt*), and GN-Δ/Δ (*asp1-Δ/asp1-Δ*) (plots a, b, c, and d, respectively). The fermentation medium was ASN medium containing asparagine and ammonium. Ammonium (○), asparagine (●) and acetic acid (▲)were monitored during fermentation. Three nitrogen depletion stages were defined. Stage 1 (dark gray): the medium contained ammonium and asparagine. Stage 2 (light gray): ammonium was depleted and only asparagine remained. Stage 3 (white): the medium contained no more nitrogen sources. For *ASP1* strains (b, c), nitrogen depletion stages 1 and 2 were simultaneous (hatched gray). For *asp1* mutants, stage 2 occurred after stage 1 and showed overproduction of acetate.

Discussion

Advantages and limitations of DNA microarrays for genotyping wild yeast strains

We applied quantitative genetics to study the genetic determinism of a quantitative trait between two wild isolates of *S. cerevisiae*, and identified a single mutation that controlled variations in their acetic acid production during alcoholic fermentation. The use of wild strains, as opposed to other studies where a laboratory strain was used as a reference (Brem *et al.*, 2002; Steinmetz *et al.*, 2002; Deutschbauer &

Davis, 2005), requires some specific comments. Indeed, wild yeasts often have very atypical genomes, with aneuploidy and/or supplementary chromosomes, translocations, etc. (Bidenne *et al.*, 1992). These genomic abnormalities often result in poor germination and complicate character segregation. These difficulties are obviously compounded in hybrids where both parents are wild yeasts. How do these 'abnormalities' affect QTL detection? (1) Reciprocal translocation kills half the progeny. The surviving spores receive the two parental alleles in equal proportions. As QTLs were tracked close to markers, translocation should not affect detection. (2) In the case of the large deletions that are frequently observed in subtelomeric regions, the presence/absence of a gene segregates as different alleles and should not disturb QTL detection. (3) Aneuploidy is more problematic. As we showed here for chromosome IX, marker identification by DNA hybridization on microarrays can be completely masked in the case of aneuploidy, where differences in hybridization intensities reflect not only rare sequence polymorphisms, but also, essentially, gene copy numbers. For some of the progeny, microarray-derived genotypes of such chromosomes may be incorrectly interpreted as being entirely inherited from one strain. In fact, the strong, apparently monoparental, marker intensity reflects inheritance of two chromosomal copies, one of which may come from the other strain. This unresolved genotyping is likely to be disastrous for QTL detection. In addition, even in the case of accurate genotyping of the aneuploid chromosome, the ability to detect any QTL located on this chromosome is reduced if the aneuploid parent strain is heterozygous, as three distinct genotypes would segregate instead of two. For all these reasons, our study is likely to have missed any QTL for acetic acid production located on chromosome IX. However, this chromosome represents only 4% of the yeast genome.

Advantages and limitations of QTL mapping for understanding the role of genetic variations in industrial processes

Our findings illustrate the importance of the exact definition of the test used to measure phenotypic values. In fact, the effect of the SNP identified here was linked to the high concentration of asparagine in the medium used for phenotyping. Despite the fact that asparagine has been used in various synthetic model grape media (Lafon-Lafourcade *et al.*, 1984; Henschke & Jiranek, 1993), it is not the sole amino acid in grape must, representing only 5–10% of the available nitrogen (Henschke & Jiranek, 1993). Although the ASN medium was previously validated by comparing acetic acid production levels with those measured in natural musts (Marullo *et al.*, 2004), its composition introduced an unsuspected bias into trait measurement. The fact that SB produced more acetic acid than GN under both conditions masked the bias introduced by asparagine in the ASN medium. Our work revealed that the acetic acid production levels of the parent strains in grape must and ASN medium were partly due to different causes. This bias emphasizes the extreme precision of phenotype detection by QTL mapping. Consequently, the relevance of phenotype testing in an industrial context should be assessed very carefully, as test conditions never match industrial situations perfectly. This study confirmed that disturbing nitrogen assimilation causes subtle changes to carbohydrate metabolism, especially acetic acid production. Compared to wild-type strains, which were readily able to use asparagine as a nitrogen source, mutated strains only used a third of the nitrogen in the medium, present in the form of NH_4^+. After this first phase (stage 1, Fig. 7), the latter suffered from nitrogen limitation, due to the low activity of the alternative asparaginase II pathway. In fact, both SB and GN had very low or null Asp3p activity, suggesting that *ASP3* genes are not active in these strains (data not shown). These findings corroborated the previously reported lack of *ASP3* expression and/or activity in various strains (Dunlop & Roon, 1975; Rossignol *et al.*, 2003). We hypothesize that the sudden decrease in glycolytic flux due to nitrogen starvation (stage 2) led to a drop in the NAD+/NADH ratio, forcing acetaldehyde through the oxidation pathway to acetate, to the detriment of the reduction pathway to ethanol. This diversion did not affect the ethanol flux significantly, as acetic acid overproduction was still very low in comparison to ethanol production.

Conclusions

The identification of QTLs linked to a physiologic trait in a eukaryotic microorganism paves the way to using more sophisticated genetic strategies to optimize industrial strains. Once a QTL has been identified, PCR/RFLP markers can be designed to track it during consecutive crosses, in a quick, inexpensive manner. Combining multiple favorable QTLs and eliminating unsuitable ones is an efficient way of obtaining new, improved strains from natural genetic resources that are currently underutilized in yeast. In fact, using PCR/RFLP markers to follow strains instead of phenotypic tests represents tremendous progress in genetic improvement.

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